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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 8 October 2002 with an application for Letters Patent number 521836 made by Tim Carroll; Ping Chen; Michelle Harnett and James Harnett.

I further certify that pursuant to a claim under Section 24(1) of the PatentsAct 1953, a direction was given that the application proceed in the name of NEW ZEALAND DAIRY BOARD.

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Dated 6 November 2003.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

COMMISSIONER OF PATENTS, TRADEMARKS & DESIGNS
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SUBSTITUTION OF APPLICANT UNDER SECTION 24

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

DAIRY PRODUCT AND PROCESS

We, Tim Carroll, Ping Chen, Michelle Harnett and James Harnett, all of Fonterra Research Centre, Dairy Farm Road, Palmerston North, New Zealand, do hereby declare this invention to be described in the following statement:

DAIRY PRODUCT AND PROCESS

FIELD OF INVENTION

The present invention relates methods of pressure treating food so as to selectively inactivate undesired microflora. More particularly the invention relates to the use of high pressure treatment to selectively inactivate undesired microflora in foods, while retaining viable desired cultures.

BACKGROUND

Many foods have relatively short shelf lives due to the unavoidable presence of contaminants such as yeasts and moulds. Such yeasts and moulds cause undesired spoilage, and often render the foods inedible.

It is known to inactivate undesirable spoilage or pathogenic organisms in food by a variety of methods, the most common of which is heating. Heat treatment can significantly improve both the safety and the keeping quality of the food. In particular the shelf life of the food can be extended.

However, properties such as the taste, texture, and nutritional quality of some foods can be compromised by a heat treatment. For example, heat-treated meat can have unacceptable cooked flavour. A heat-treated cultured milk product such as yoghurt does not contain a live bacterial culture, as the culture is inactivated by the treatment.

The fermentation of certain foods such as milk, vegetables or meat by bacteria such as lactic-acid bacteria has long been recognised as a method of imparting an improved keeping quality, as well as an improved flavour or texture. Typical food-spoilage and pathogenic bacteria are unable to thrive in the acidic environment of the fermented food, although yeasts and moulds can survive, and as such become the dominant spoilage organisms.

Historically, it was recognised that holding over a portion of a previous fermentation to start a subsequent one was a way of maintaining the quality of the product. With advances in microbiology coupled with production at industrial scale, this method was replaced by isolation, production, and supply of specific starter cultures as a method of ensuring product consistency and fermentation reliability (Caplice & Fitzgerald, Everson et al).

It is also known that preparation of the fermentable substrate to inactivate undesirable microorganisms before inoculating with the desired starter culture, improves the reliability of the fermentation.

It was recognised at the beginning of the last century, that bacteria used for the fermentation of products such as yoghurt, are of benefit to human health if consumed alive It is now recognised that certain cultures of live micro-organisms, defined as probiotic, exert health benefits beyond basic nutrition upon ingestion in certain numbers. (Holzapfel et al). It is known to add these probiotic bacteria to foods (Lee and Salaminen) for delivery by ingestion. However, It is not possible to deliver these bacteria in sufficient numbers in a food that is subsequently heat-treated to meet a food safety or spoilage objective.

In cases such as those above, it is desirable to retain bacterial cultures in a food while inactivating contaminants.

It is an object of the present invention to provide an improved or alternative method of treating a food product, and / or to go at least some way to overcoming the problems encountered with the prior art.

SUMMARY OF INVENTION

In one aspect the invention broadly consists of a method of selectively inactivating undesired microflora in a food while retaining a viable culture comprising the following steps:

- selecting a food containing at least one strain of a culture, said strain capable of surviving a pressure treatment at a predetermined pressure, and
- subjecting the food to that pressure.

Preferably the culture is selected such that at least one strain survives a pressure treatment at the predetermined pressure.

In a second aspect the invention broadly consists of a method of treating a cultured dairy product to reduce numbers of undesired microflora, comprising the steps:

- selecting a cultured dairy product containing at least one strain of culture, said strain capable of surviving a pressure treatment at a predetermined pressure within a predetermined pH range, and
- subjecting the cultured dairy product to that pressure within that pH range.

Preferably the pressure treatment extends the useful shelf life of the cultured dairy product.

Preferably the cultured dairy product is subjected to a pressure of at least 350MPa.

More preferably the cultured dairy product is subjected to a pressure of at least 400MPa.

Alternatively cultured dairy product is subjected to a pressure of at least 400 MPa for at least about 1 minute.

Alternatively cultured dairy product is subjected to a pressure of at least 200MPa.

In the preferred embodiment, the cultured dairy product is subjected to the pressure treatment at a pH of between 3.0 and 8.0.

Preferably the pH is between 3.6 and 4.8.

Most preferably the pH is between 4.0 and 4.6.

Preferably the cultured dairy product is pressure treated at a temperature in the range 0°C-40°C.

Most preferably the cultured dairy product is pressure treated at a temperature in the range 0°C-20°C.

In a preferred embodiment, the cultured dairy product is pressure treated after packaging.

A preferred cultured dairy product used in the invention is yoghurt.

Alternative cultured dairy products used in the invention may be selected from yoghurt drinks, dairy desserts, cottage cheese, cream cheese and cultured beverages.

Preferred strains of culture used in this aspect of the invention are selected from: *Lactobacillus rhamnosus* HN067 AGAL deposit number NM97/01925 dated 11 February 1998, *Lactobacillus acidophilus* HN017 AGAL deposit number NM97/09515 dated 18 August 1997, *Bifidobacterium lactis* HN019 AGAL deposit number NM97/09513 dated 18 August 1997, *Streptococcus thermophilus* St10, *Streptococcus thermophilus* St49, *Lactobacillus helveticus* Lh1, *Lactobacillus helveticus* Lh5001, *Lactobacillus delbrukeii subsp bulgaricus* Lb1, Rhodia MY900 (commercially sold by Rhodia under the trade mark "MY900"), *Bifidobacterium* Bb12 (commercially sold by Nestle under the trade mark "Bb12") and *Bifidobacterium* Wisby 420 (commercially sold by Wisby under the trade mark "420"). The strains identified as St10, St49, Lh1, Lh5001 and Lb1 are

commercially available from the Fonterra Research Centre, Palmerston North, New Zealand.

In a third aspect the invention broadly consists in a method of selectively inactivating undesired microflora in a food while retaining a viable culture comprising the following steps:

-selecting a food containing at least one strain of a culture, said strain capable of fermenting the food after a pressure treatment at a predetermined pressure, and

- subjecting the food to at least that pressure.

The food used in the method of this aspect of the invention may be selected from meat, meat products (such as salami or sausage), vegetables and vegetable products (such as olives, pickles, cabbage, soy beans, sauerkraut), cheese and cheese analogues.

In a fourth aspect, the invention consists in a method of treating a food, comprising the steps:

- selecting a food containing at least one probiotic strain of a culture, said probiotic strain capable of surviving a pressure treatment at a predetermined pressure, and

- subjecting the food to at least that pressure.

In one alternative of the invention, the probiotic is used to ferment the food.

In another alternative the probiotic is added to the food before pressure treatment.

Probiotic strains used in the invention may be selected from strains of *Bifidobacterium*.

Preferably probiotic strains selected from used in the invention are selected from strains of *Bifidobacterium lactis*.

Preferred probiotic strains used in the invention are selected from *B. lactis* HN019 AGAL deposit number NM 97/09513 dated 18 August 1997, and *Bifidobacterium* sold under the trade names Bb12 (Nestle) and Wisby 420.

Alternatively probiotic strains used in the invention are selected from strains of *Lactobacillus.*

Preferably probiotic strains used in the invention are selected from strains of *Lactobacillus acidophilus.*

Most preferably a probiotic strain used in the invention is *Lactobacillus acidophilus* HN017 AGAL deposit number NM 97/09515 dated 18 August 1997.

Preferably the pressure is at least 250MPa.

More preferably the pressure is at least 300MPa.

Even more preferably the pressure is at least 400MPa.

Alternatively the pressure treatment is at least 400Mpa for at least about one minute.

Alternatively the pressure is at least 500MPa.

Alternatively the pressure treatment is 500MPa for at least about one minute.

In a fifth aspect, the invention consists in a method of treating a food comprising the following steps:

- selecting a food containing at least one strain of protective culture, said strain capable of surviving a pressure treatment at a predetermined pressure, and
- subjecting the food to at least that pressure.

Preferably the protective culture is selected from those used in cultured dairy foods, fermented foods, cooked meats, vegetables, salads, cook-chilled foods, ready-to-eat foods.

The invention also consists in a food treated by the methods described herein.

In a sixth aspect the invention consists in the use of at least one bacterial strain in a food to be subjected to a pressure treatment at a predetermined pressure such that undesired microflora are inactivated and the strain used survives, said bacterial strain being selected from: *Lactobacillus rhamnosus* HN067 AGAL deposit number NM97/01925 dated 11 February 1998, *Lactobacillus acidophilus* HN017 AGAL deposit number NM97/09515 dated 18 August 1997, *Bifidobacterium lactis* HN019 AGAL deposit number NM97/09513 dated 18 August 1997, *Streptococcus thermophilus* St10, *Streptococcus thermophilus* St49, *Lactobacillus helveticus* Lh1, *Lactobacillus helveticus* Lh5001, *Lactobacillus delbruekeii subsp bulgaricus* Lb1, Rhodia MY900, and *Bifidobacterium* sold under the trade names Bb12 (Nestle) and Wisby 420 (Wisby).

The invention may also broadly be said to consist in any alternative combination of features as described or shown in the accompanying examples. Known equivalents of these features not expressly set out are nevertheless deemed to be included.

DETAILED DESCRIPTION

As mentioned herein, references to "pressure treatment" or "UHP treatment" mean ultra high-pressure treatments. Such treatments are generally accepted as pressure treatments using pressures of at least 100MPa. This is also known in the art as "high pressure", "high hydrostatic pressure" (HHP) or "high pressure proccessing" (HPP).

As mentioned herein, references to "food" or "foodstuffs" include, for example yoghurts, yoghurt drink, Kefir, cheeses, milk, dairy products, dairy desserts, fruit juices, sport drinks, meat (for example salami), vegetables and the like.

As mentioned herein, references to "undesired microflora" refer to contaminants such as yeasts and moulds, spoilage organisms, pathogens, naturally present bacteria, and starter organisms that have completed their function.

As mentioned herein, references to "protective culture" refer to a culture that produces a metabolite, such as bacteriocin or acid, which exhibits antimicrobial activity.

As mentioned herein, references to "probiotics" refer to strains of bacteria with health promoting and immune-enhancing properties. Such strains have an ability to survive in the intestine and gut, and are well known in the art.

A major advantage of UHP treatment over methods of microbial inactivation treatments is its non-invasive nature. With appropriate equipment, foodstuffs and the like can be treated in the same containers that they are to be distributed to the consumer in. For example, set or stirred yoghurt can be treated in pottles and cultured dairy drinks can be treated in their bottles.

However, in situations where the desired microorganisms (e.g. cultures) and the contaminants are in the same sealed container, a method of selective killing was required.

Surprisingly, we have discovered that it is possible to subject a food to a UHP treatment that inactivates spoilage, pathogenic or other unwanted microorganisms, but in which select desirable bacteria survive the treatment in useful numbers. These desirable bacteria could be typical yoghurt or probiotic cultures that offer a health benefit if consumed live or protective cultures that can inhibit contaminating microorganisms. In general, these desirable bacteria could be starter cultures for fermentation that can be retained live after a treatment that inactivates undesirable microorganisms, to improve the reliability of the fermentation, or the flavour, texture or colour of the fermented product.

Thus the invention can be used to produce a food containing a desired culture but without active microflora. The invention offers the ability to preserve the quality of foods containing a live culture that is of significant commercial value.

In particular we have found that we can produce cultured dairy products (such as yoghurt containing abundant live cultures) having an extended shelf life due to protection against spoilage by unwanted yeasts and moulds.

The invention can be used to produce a range of food products containing an abundant live probiotic culture, with an improved keeping quality due to protection against spoilage.

Furthermore, we can produce foods containing a live protective pressure resistant culture that is capable of inactivating spoilage microorganisms. This process can be used to improve the safety and keeping quality of certain foods.

An important factor in successfully performing a method according to the invention is identifying which strains of the desired culture are capable of surviving pressure treatment at a pressure that inactivates undesired microflora.

One method of identifying appropriate cultures for use in the present invention is to inoculate strains into a food and then treat the food at a pressure suitable for controlling contamination by yeasts and moulds, for example 350 MPa for 5 minutes. Strains that survive the test-pressure treatment in useful numbers are identified as strains appropriate for use in the present invention.

Once these suitably resistant strains of culture have been identified, a food product can be manufactured containing that strain of culture. Alternatively, off the shelf food products know to contain pressure resistant bacteria could be identified. Said food products can then be subjected to pressure treatment under conditions which are known to inactivate undesired microflora, but allow survival of the selected strains of culture.

Some starter cultures can produce anti-microbial metabolites or bacteriocins, that impart a more specific protection against certain microorganisms (Brul & Coote, Holzapfel et al, Caplice & Fitzgerald). The most prevalent of these compounds is nisin, a bacteriocin against most Gram-positive bacteria produced by *Lactococcus lactis*, and approved for use as a food ingredient by the Food and Drug Administration and World Health Organisation in 1969 (Helander et al). The use of these compounds is preferable to the use of chemical preservatives, as they are natural in origin. However, it is more desirable to add a bacteriogenic culture as a starter or starter adjunct to a food rather than the bacteriocin itself where the culture has GRAS status. (Caplice and Fitzgerald). Such cultures are commercially available, for example those sold under the trade marks of Bioprofit or Microgard.

Examples of the use of such protective cultures are:

- The addition of a culture of *Lactobacillus sake* to Bologna-style sausage, which inhibits the growth of *Listeria monocytogenes* (Abee).
- The addition of a *Pediococcus acidilactici* culture to fermented sausages, which inhibits the growth of *Listeria monocytogenes* (Yousef).
- The use of a probiotic and bioprotective strain of *Lactobacillus rhamnosis* in the fermentation of sausages (Erkkila).
- The inclusion of a protective culture to improve the safety of cook-chill foods (Rodgers).

Using the methods taught in the present invention, it is now possible to inactivate undesired microflora whilst retaining the protective cultures in the food for enhanced food safety. The remaining protective culture (or cultures) would then inactivate undesired microflora that survives pressure treatment.

EXAMPLES

In some of the following examples, typical contaminants were deliberately added to the food products. The food products were then treated according to the methods of the invention.

The methods used to enumerate the bacteria in the following examples are summarised in Table 3. All cultures were obtained from the Fonterra Research Centre Culture Collection ("FRCCC") in Palmerston North, New Zealand, with the exception of the cultures sold under the trade marks "Bb12" (Nestle) and "Wisby 420" (Wisby). The contaminant cultures used in the examples and their respective origins are summarised in Table 4.

Example 1: A cultured dairy product containing an abundant viable culture

A 10% reconstituted skim milk (RSM) substrate was inoculated with 1% of a culture of *Streptococcus thermophilus* St-10 (FRCCC) and fermented overnight at 37°C. The pH of the cultured skim milk was adjusted to 4.4 by addition of lactic acid, and intentionally contaminated by spiking at 1.8×10^6 cfu/mL (Table 3) with a *Yarrowia lipolytica* yeast (Table 4). The contaminated cultured milk so produced was then treated by applying a pressure of 400 MPa for 5 minutes at 10°C. This process produced a product with no detectable contaminating yeast (>5-log inactivation), while retaining a starter culture count of 3.3×10^7 cfu/mL (M17 agar, Table 3).

Example 2 : A cultured dairy product containing an abundant viable culture

A 10% RSM substrate was inoculated with 1% of a culture of *Lactobacillus helveticus* Lh-5001 (FRCCC) and fermented overnight at 37°C. The pH of the cultured skim milk was adjusted to 4.4 and intentionally contaminated by spiking to 3.1×10^6 cfu/mL (Table3) with a Penicillium mould (Table 4). The contaminated cultured milk so produced was then treated by applying a pressure of 400 MPa for 5 minutes at 10°C. This process

produced a product with no detectable contaminating mould, while retaining a starter culture count of 1.3 x 10^8 cfu/mL (MRS agar, Table 3).

Example 3 : A cultured dairy product containing an abundant viable culture

A 10% RSM substrate was inoculated with 1% of a culture of *Lb. delbruekii sub-species bulgaricus* Lb-1 (FRCCC) and fermented overnight at 37°C. The pH of the cultured skim milk was adjusted to 4.4 , and intentionally contaminated by spiking at 9.3x10^7 cfu/mL (Table 3) with a *Debromyces hanseii* yeast (Table 4). The contaminated cultured milk so produced was then treated by applying a pressure of 350 MPa for 5 minutes at 10°C. This process produced a product with no detectable contaminating yeast, while retaining a starter culture count of 7.6 x 10^7 cfu/mL (MRS agar, Table 3).

Example 4 : Cultured dairy product with no significant live viable culture [control example]

A 10% RSM substrate was inoculated with 1% of a culture of *S. thermophilus* St-1 (FRCCC) and fermented overnight at 37°C. The pH of the cultured skim milk was adjusted to 4.4 by addition of lactic acid, and intentionally contaminated by spiking at 3.5x10^6 cfu/mL (Table 3) with a pink yeast (Table 4). The contaminated cultured milk was then treated by applying a pressure of 400 MPa for 5 minutes at 10°C. This process produced a product with no detectable contaminating yeast (>5-log inactivation). However, the starter culture was reduced from 4.4 x 10^7 cfu/mL to 6.8 10^2 cfu/mL (>4-log inactivation) by the pressure treatment. On this basis, the invention cannot be performed by using strain St-1 and pressure-treatment of 400 MPa or higher for 5 or more minutes at pH 4.4.

Example 5 : A yoghurt containing an abundant viable culture

A yoghurt milk made up with 7.0% skim milk powder (SMP) and 7.5% whole milk powder (WMP), was heated to 55°C and homogenised at 150/50 bar. The homogenised

milk was then heated to 90°C in a steam-heated waterbath and held at that temperature for 10 minutes. After rapid cooling to 42°C, the milk was inoculated with 1% of a culture of *S. thermophilus* St-10 and *Lb. delbruekii sub-species bulgaricus* strain Lb-5033 (FRCCC), then fermented at 42°C to a pH of 4.4, whereupon it was cooled to 4°C. The resulting yoghurt was intentionally contaminated by spiking at $4.4x10^6$ cfu/mL with a Penicillium mould and pink yeast. The contaminated yoghurt was then treated by applying a pressure of 450 MPa for about 1 minute at 15°C. This process produced a product with no detectable contaminating yeast (>6-log inactivation), while retaining a starter culture count of 3.0×10^8 cfu/mL (M17 agar, Table 3) and 1.4×10^8 cfu/mL (MRS agar, Table 3).

Example 6 : A fruit yoghurt containing an abundant viable culture

7% of a sterile fruit puree was added to yoghurt made as in Example 5. It was intentionally contaminated by spiking at $5.4x10^6$ cfu/mL with a Penicillium mould and pink yeast. The contaminated yoghurt was then treated by applying a pressure of 450 MPa for about 1 minute at 15°C. This process produced a product with no detectable contaminating yeast (>6-log inactivation), while retaining a starter culture count of 6.6×10^8 cfu/mL (M17 agar, Table 3).

Example 7 : A yoghurt drink containing an abundant viable culture

The yoghurt, produced as in Example 5, was made up to a final formulation of 8% sugar, 1% protein and 0.4 % carboxymethylcellulose .The pH of the yoghurt was then adjusted to 4.0 by addition of a citric/lactic acid solution and homogenised at 200 bar. The resulting yoghurt drink was intentionally contaminated by spiking at $7.9x10^6$ cfu/mL with a Penicillium mould and pink yeast. The contaminated yoghurt drink was then treated by applying a pressure of 450 MPa for about 1 minute at 15°C. This process produced a product with no detectable contaminating yeast (>6-log inactivation), while retaining a starter culture count of 2.1×10^8 cfu/mL (M17 agar, Table 3).

Example 8 : A yoghurt containing an abundant viable culture

A yoghurt milk made up with 7.0% SMP and 7.5% WMP, was heated to 55°C and homogenised at150/50 bar. The homogenised milks were then heated to 90°C in a steam-heated waterbath and held at that temperature for 10 minutes. After rapid cooling to 42°C, the yoghurt milk was inoculated with 1% each of *S. thermophilus* St-10 and *Lb. delbruekii sub-species bulgaricus* strain Lb-1 and fermented at 42°C to a pH of 4.0, whereupon it was cooled to 4°C. The yoghurt was contaminated by spiking at $3.0x10^6$ cfu/mL with a Penicillium mould and a pink yeast, and treated by applying a pressure of 450 MPa for about 1 minute at 15°C. This process produced a product with no detectable contaminants (>6-log inactivation), while retaining a starter culture count of 1.8×10^8 cfu/mL (M17 agar, Table 3) and 4.1×10^7 cfu/mL (MRS agar, Table 3).

Example 9 : A yoghurt with no significant viable culture after treatment at a deliberately low final pH

A yoghurt milk made up with 7.0% SMP and 7.5%WMP, was heated to 55°C and homogenised at 150/50 bar. The homogenised milks were then heated to 90°C in a steam-heated waterbath and held at that temperature for 10 minutes. After rapid cooling to 42°C, the yoghurt milk was inoculated with 1% each of *S. thermophilus* St-10 and *Lb. delbruekii sub-species bulgaricus* strain Lb-1 and fermented at 42°C to a pH of 3.6, whereupon it was cooled to 4°C. The yoghurt was intentionally contaminated by spiking at $3.0x10^6$ cfu/mL with a Penicillium mould and a pink yeast , and treated by applying a pressure of 450 MPa for about 1 minute at 15°C. This process produced a product with no detectable contaminants (>6-log inactivation). However, the starter culture count was reduced to 1.3×10^4 cfu/mL, from 3.2×10^8 cfu/mL (M17 agar, Table 3).

Example 10: A yoghurt containing a live probiotic culture

A yoghurt milks made up with 7.0% SMP and 7.5% WMP, was heated to 55°C and homogenised at 150/50 bar. The homogenised milks were then heated to 90°C in a steam-heated water bath and held at that temperature for 10 minutes. After rapid cooling to 42°C, the yoghurt milk was inoculated with 1% *B. lactis* HN019, 0.25% ST10 and 0.25% Lb TH (*Lb. delbruekii sub-species bulgaricus* TH, FRCCC). The yoghurt milk was incubated at 38°C overnight (16 h) to a pH of 4.0., removed from the incubator and cooled to approximately 25°C. It was then smoothed by passing it through a homogeniser at 0 bar and filled into 375mL Polyethylene (PET) bottles and stored at 5°C. The yoghurts were pressure-treated at 375 MPa for 5 minutes.

An untreated yoghurt sample was retained as a control.

After four weeks storage at 4°C, the yoghurts contained a live probiotic culture (*B. lactis* HN019) of 1.3×10^7 cfu/mL (RBA agar, Table 3), with no contaminating yeasts and moulds.

In contrast, although the untreated control sample contained a live probiotic culture of 2.0×10^7 cfu/mL, it was contaminated with 1.2×10^3 cfu/mL of yeasts and moulds after 4 weeks at 4°C.

Example 11: A yoghurt containing a live probiotic culture

A yoghurt formulated as 7.0% SMP, 7.5%WMP, 0.1% pectin and 0.4% starch was prepared as for Example 10. The yoghurt was pressure-treated at 375 MPa for 5 minutes, and untreated yoghurt was retained as a control. After the treated sample was stored at 4°C for 4 weeks, it contained a live probiotic culture of 5.6×10^7 cfu/mL (RBA agar, Table 3), with no contaminating yeasts and moulds. In contrast, although the untreated control sample contained a live probiotic culture of 6.2×10^7 cfu/mL(after 4 weeks at 4°C) it was contaminated with 3.2×10^2 cfu/mL of yeasts and moulds.

Example 12 : A dairy product containing a live probiotic culture

A 10% RSM substrate was inoculated with 1% of a culture of *L. acidophilus* HN017 (FRCCC) and fermented overnight at 37°C. The pH of the cultured skim milk was adjusted to 4.4, and contaminated by intentionally "spiking" a *Cladosporium* mould at $1.5x10^5$ cfu/mL (Table4). The contaminated cultured milk so produced was then treated by applying a pressure of 200 MPa for 5 minutes at 10°C. This process produced a product with no detectable contaminating mould, while retaining a starter culture count of 2.8×10^8 cfu/mL (MRS agar, Table 3).

Example 13: A direct acidified milk drink containing a live probiotic culture

A direct acidified milk drink was made by blending Carboxymethylcellulose (CMC) with sugar, and then dispersing it in 1.5 L warm (55°C) water. WMP was separately dispersed in 1.5 L warm (55°C) water and then mixed with the CMC-sugar solution. More sugar was added to give a final formulation of 8% sugar, 3.2% WMP and 0.4 % CMC. The pH of the milk drink was then adjusted to 4.0 by addition of a citric/lactic acid solution, homogenised at 200 bar, and sterilised at 85°C in steam bath for 5 minutes. The drink was then chilled and inoculated with $5.7x10^7$ cfu/mL (RCA agar, Table 3) of *Bifidobacterium* (Wisby strain 420). The probiotic drink produced was then treated by applying a pressure treatment of 350 MPa for 5 minutes at room temperature (15 °C). A drink containing live probiotic *Bifidobacterium* at the level of $4.7x10^7$ cfu/mL (RCA agar, Table 3) was so produced.

Example 14: A direct acidified milk drink containing no significant live probiotic culture [control example]

The direct-acidified milk drink made as in Example 13 was inoculated with at $4.9x10^7$ cfu/mL (MRS agar, Table 3) of *L. casei* Yakult Shirota strain. The sample was treated at

a pressure of 350 MPa for 5 minutes at 15 °C. This process produced a product with a count of only 240 cfu/mL (5.3-log reduction) of the *L.casei* probiotic culture after the pressure treatment. A useful dose of this probiotic strain cannot be delivered in this product format under these pressure-treatment conditions.

Example 15: An orange juice containing a live probiotic culture

Australian navel oranges were squeezed to produce an orange juice of pH 3.48 and total solids of 10.6 %. *B. lactis* strain HN019 was inoculated into the juice at $1.1x10^8$ cfu/mL (RCA agar, Table 3) and the juice was intentionally contaminated by spiking at $2.2x10^7$ cfu/mL with pink yeast. The contaminated probiotic orange juice was than treated at a pressure of 350 MPa for 5 min at 15 °C. This process produced a product with no detectable contaminating pink yeast (> 6-log inactivation) while retaining a probiotic culture count of $1.1x10^8$ cfu/mL (RCA agar, Table 3).

Example 16: An orange juice containing a live probiotic culture and unacceptable levels of contamination

The contaminated orange juice made as in Example 15 was treated at a pressure of 300 MPa for 5 minutes at 15°C. Although, the product retained a probiotic culture count of $1.1x10^8$ cfu/mL (RCA agar, Table 3), it was contaminated by 3.8×10^3 cfu/mL of pink yeast.

Example 17: An orange juice containing a live probiotic culture

The contaminated orange juice made as in Example 15 was treated at a pressure of 600 MPa for 5 minutes at 15 °C. This process produced a product with no detectable contaminating Pink yeast (> 6-log inactivation) while retaining a probiotic culture count of $4.1x10^6$ cfu/mL (RCA agar, Table 3).

Example 18: Method of strain selection

Potential probiotic strains were screened for suitability in the production of a pressure-treated direct-acidified milk drink as follows. A number of commercially available probiotic strains were inoculated into the milk drink and then treated at a pressure suitable for controlling contamination by yeasts and moulds, specifically 350 MPa for 5 minutes. The results of the selection method are shown in Table 1. Neither of the *Lactobacillus* strains evaluated survived the pressure treatment in sufficient numbers. However two of the *Bifidobacterium* strains sold under the trade marks "Bb12" (Nestle) and "Wisby 420" (Wisby), are not appreciably inactivated by the test-pressure treatment. Either of these strains is suitable for delivery in a direct-acidified milk drink that is protected by a pressure treatment, against contamination by yeasts and moulds.

Table 1: Method of selecting a suitable probiotic strain

Species	Strains	Count (cfu/mL)		$> 10^6$cfu/mL
		Before treatment	After treatment	
L. casei	shirota - Yakult	4.90×10^7	2.40×10^1	no
L. johnsonii	Lc-1- Nestle	7.80×10^7	9.00×10^1	no
Bifidobacterium	536 - Morinaga	1.30×10^8	9.00×10^1	no
Bifidobacterium	Bb12 - Nestle	6.20×10^7	5.20×10^7	yes
Bifidobacterium	420 - Wisby	5.70×10^7	3.30×10^7	yes

Example 19: Method of strain selection

Potential probiotic strains were screened for suitability in the production of a pressure-treated probiotic orange juice as follows. Two commercially available probiotic strains were inoculated into the juice and then treated at a pressure suitable for controlling contamination by spoilage bacteria, specifically 600 MPa for 5 minutes. The results of

the selection method are shown in Table 2. Both *Bifidobacterium* strains survive the pressure-treatment in sufficient numbers for delivery in an orange juice that is protected by a pressure-treatment, against spoilage by yeasts, moulds and bacteria.

Table 2: Method of selecting a suitable probiotic strain

Species	Strains	Count(cfu/mL)		$> 10^6$ cfu/mL
		Before treatment	After treatment	
Bifidobacterium	Bb12 - Nestle	1.1×10^8	4.1×10^6	yes
Bifidobacterium	420 - Wisby	7.0×10^7	1.2×10^6	yes

Example 20: A low pH sports drink with a live probiotic culture

A whey protein isolate sport drink (4.8% whey protein isolate, protein, 0% fat and 10% carbohydrate), with a pH of 3.4, was inoculated with *Bifidobacterium lactis* strain HN019 at 9.2×10^7 cfu/mL (RCA agar, Table 3) and intentionally contaminated by spiking at 4.5×10^5 cfu/mL with pink yeast. The contaminated probiotic low pH sport drink was than treated at a pressure of 400 MPa for 5 minutes at 15 °C. This process produced a low pH sport drink with no detectable contaminating Pink yeast (> 5.6-log inactivation) while retaining a probiotic culture count of 2.7×10^7 cfu/mL (RCA agar, Table 3).

Example 21: A nutrition shake with a live probiotic culture

A Myoplex French vanilla nutrition shake made by EAS, USA, (20 g protein (whey and soy protein isolates, 4.6 g fat and 20 g carbohydrate per 330mL serving). was inoculated with *Bifidobacterium lactis* strain HN019 at 5.5×10^7 cfu/mL and intentionally contaminated by spiking at 5.4×10^5 cfu/mL with Pink yeast. The contaminated probiotic neutral pH sport drink was than treated at a pressure of 500 MPa for 5 minutes at 15 °C. This process produced a neutral pH sport drink with no detectable contaminating Pink yeast (> 5.7-log inactivation) while retaining a probiotic culture count of 4.8×10^7 cfu/mL.

Example 22 : A yoghurt containing an abundant viable culture

A yoghurt milk made up with 7.0% SMP and 7.5% WMP, was heated to 55°C and homogenised at 150/50 bar. The homogenised milk was then heated to 90°C in a steam-heated water bath and held at that temperature for 10 minutes. After rapid cooling to 42°C, the yoghurt milk was inoculated with 1% of a starter culture sold by Rhodia under the trade mark "MY900", and fermented at 42°C to a pH of 4.1, whereupon it was cooled to 4°C. The yoghurt was pressure-treated at 400 MPa for 5 minutes (A treatment selected based on complete inactivation of contaminating yeasts and moulds, as in earlier Examples). The treated yoghurt had a viable culture of 7.9×10^7 cfu/g (M17 agar, Table4).

Example 23 : A yoghurt containing an abundant viable culture

A yoghurt milk, made up with 12.0% whole milk powder (WMP) and 1.7% of whey protein concentrate was heated to 90°C in a steam-heated water bath and held at that temperature for 10 minutes. After cooling to 42°C, the yoghurt milk was inoculated with a starter culture sold by Rhodia under the trade mark "MY900" (commercial culture), and fermented at 42°C for 6 hours, to a pH of 4.4, Whereupon it was cooled to 4°C. The contaminated yoghurt was then pressure-treated at 450 MPa for about 5 minute at 15°C. Based upon previous examples, this treatment is sufficient to inactivate typical dairy yeasts and moulds. This process produced a product with a starter culture count of 3.0×10^7 cfu/mL (M17 agar, Table 3).

Enumeration of culture

An appropriate amount of sample was added to diluent to produce a 10^{-1} dilution. Then, subsequent dilutions were prepared serially to plate out a range from 10^{-1} to10^{-7} for each sample. The dilutions were then plated onto appropriate agars for each species (Table 3).

The agars were selected for efficient recovery of the test species, and prepared according to the manufacturers instructions. The species and corresponding method used are summarised in Table 3.

5 **Table 3: A Summary of the Methods used for Microbiological Enumeration**

Culture	Agar used	Supplier	Summary of conditions
S. thermophilus	M17	Difco (Cockeysville, USA)	37°C, 48h, aerobic
Lb. delbruekii subsp bulgaricus	MRS	Difco	37°C, 48h, Anaerobic
L. helveticus			
Yeasts and moulds	YGCA	Merck Darmstadt, Germany	25°C, 5 days
Bifidobacterium	RCA	Merck	37°C, 3 days, Anaerobic
L. rhamnosus	MRS	Difco	37°C, 3 days, Anaerobic
L. johnsonii			
L. para-casei			
L. casei			
**Bifidobacterium* differential	RBA (Ribose BCP agar)	Made at Fonterra Research Centre	37°C, 3 days Anaerobic

RBA agar reference: Raffinose-Bifidobacterium (RB) agar, a new selective medium for Bifidobacteria', R. Hartemink et al, Journal of Microbial Methods 27 (1996) 33-43

Table 4 : A summary of the yeast and mould contaminants used in the examples

Strain	Origin	Source	Description
Yarrowia lipolytica	Yeast isolate from dairy product	FRC* Starter Group culture collection	Contaminant from a dairy product
Unknown	'Pink yeast' Isolated from a dairy product	FRC Starter Group culture collection	Contaminant from dairy product
Candida famata	Yeast isolate from dairy product	FRC Starter Group culture collection	Contaminant from a dairy product
Candida parapsilosis	Yeast isolate from dairy product	FRC Starter Group culture collection	Contaminant from a dairy product
Debramyces Hanseii	Yeast isolate from dairy product	FRC Starter Group culture collection	Contaminant from a dairy product
Geotrichum candidum	Mould isolate from dairy product	FRC Starter Group culture collection	Contaminant from a dairy product
Cladosporium	Mould isolate from dairy product	FRC Starter Group culture collection	Contaminant from a dairy product
Penicillium	Mould isolate from dairy product	FRC Starter Group culture collection	Contaminant from a dairy product

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